

# Procyanidin B3, an inhibitor of histone acetyltransferase, enhances the action of antagonist for prostate cancer cells via inhibition of p300-dependent acetylation of androgen receptor

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Increasing evidence suggests that AR (androgen receptor) acetylation is critical for prostate cancer cell growth. In the present study, we identified Pro-B3 (procyanidin B3) as a specific HAT (histone acetyltransferase) inhibitor. Pro-B3 selectively inhibited the activity of HATs, but not other epigenetic enzymes. Pro-B3 substantially inhibited the p300-mediated AR acetylation, both *in vitro* and *in vivo*. Pro-B3 inhibited both p300-dependent and agonist-induced AR transcription. We demonstrate that the p300-mediated AR acetylation is critical for the hormone responsiveness of AR. Interestingly, B3 treatment efficiently enhanced the antagonist activity of flutamide through suppression

of p300 HAT activity, demonstrating that relative p300 activity is critical for the antagonist action. Finally, Pro-B3 treatment inhibited acetylation-dependent prostate cell proliferation and expression of cell-cycle control genes, subsequently increasing cell death, indicating the functional importance of AR acetylation for prostate cancer cell growth.

**Key words:** acetylation, androgen receptor, histone acetyltransferase, procyanidin b3, prostate cancer.

## INTRODUCTION

AR (androgen receptor) plays an important role in normal male development, as well as prostate cancer cell growth and progression [1]. AR function is modulated by post-translational modifications such as acetylation, ubiquitylation, SUMOylation and phosphorylation [2–5]. p300/CBP [CREB (cAMP-response-element-binding protein)-binding protein]-mediated AR acetylation is a key post-translational modification, regulating AR growth control and hormone responsiveness in prostate cancer cell lines [6]. Tip60 is also known to regulate the transcription of AR in an androgen-independent manner via enhancing nuclear translocation of AR [7,8]. There is ample evidence to suggest that the ligand-induced transcriptional activity of an acetylation mimetic mutant, AR<sub>K630Q</sub>, and a somatic mutation identified in prostate cancer patients at the AR acetylation site, AR<sub>K630T</sub>, is greater than that of wild-type AR [2,6,9]. These AR acetylating-site mimetics also show enhanced responsiveness at lower DHT (dihydrotestosterone) concentrations than wild-type AR [6]. In addition, the DHT antagonist flutamide antagonizes DHT-induced wild-type AR activity; however, the AR acetylation mimetics are relatively resistant, suggesting that AR acetylation might be an ideal target for novel tumour therapies [6,7,9].

As suggested in previous studies, the development of HATi [HAT (histone acetyltransferase) inhibitors] from dietary compounds, such as garcinol, curcumin, anacardic acid and EGCG (epigallocatechin-3-gallate), are current therapeutic goals [10–13]. Garcinol inhibits p300 and PCAF (p300/CBP-activating factor) *in vitro* and *in vivo* [10], both anacardic acid and EGCG inhibit Tip60, as well as p300 and PCAF [12,13], and curcumin inhibits p300 and PCAF [11]. Therefore p300 is the common target of these HATi. Since either mutation or amplification of p300 has been reported in colorectal, breast and prostate cancer, the dysregulation of protein acetylation by p300 is presumably associated with oncogenesis [14,15]. Even though the antitumour activities of these HATi are well documented, it is still unclear whether HATi-mediated inhibition of p300 activity is correlated with cancer cell proliferation.

During the effort to identify natural anti-HAT substances, we found that procyanidins from grape seed extracts possess a potent anti-HAT activity. Grape seed is a rich source of procyanidins, polymer chains of flavonoids such as catechins [16]. Grape seed procyanidins are known to possess both chemopreventative and antiproliferative effects on breast, prostate, skin and colorectal cancer cells [17–20]. Although procyanidin is known to inhibit the growth of prostate cancer cells, it is unclear how procyanidin suppresses prostate cancer cell survival. In the present study, we

Abbreviations used: aa, amino acids; AR, androgen receptor; ARE, androgen-response element; CBP, CREB (cAMP-response-element-binding protein)-binding protein; ChIP, chromatin immunoprecipitation; DHT, dihydrotestosterone; DTT, dithiothreitol; EGCG, epigallocatechin-3-gallate; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; GST, glutathione transferase; HAT, histone acetyltransferase; HATi, HAT inhibitor(s); HDAC, histone deacetylase; HEK, human embryonic kidney; HMT, histone methyltransferase; IP, immunoprecipitation; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; NE, nuclear extract; PCAF, p300/CBP-activating factor; Pro-B3, procyanidin B3; PSA, prostate-specific antigen; RT, real-time; siRNA, small interfering RNA.

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demonstrate that Pro-B3 (procyanidin B3) exhibits the strongest inhibition against p300 HAT of all catechin derivatives. We show that Pro-B3 suppresses the androgen-dependent transcription of AR. Furthermore, we show that Pro-B3 suppresses p300-enhanced AR transcription in an acetylation-dependent manner. Finally, we demonstrate the enhanced antagonistic activity of Pro-B3, which may aid in the development of effective chemopreventive therapeutics.

## EXPERIMENTAL

### Cell culture, reagents and plasmids

Human LNCap and PC-3 cells were maintained in RPMI 1640 (Gibco/BRL) supplemented with 10 % FBS (fetal bovine serum; Hyclone). Cells grown in six-well plates were supplemented with fresh medium 2 h before transfection with 1.5 µg of DNA, according to the manufacturer's protocol for Effectin transfection (Qiagen). After 2–3 h of incubation, cells were treated with medium supplemented with charcoal-dextran-treated FBS, containing either 1 nM synthetic androgen R1881 (NEN/Life Sciences Products) or 1 µM antagonist, including flutamide. Cell lysates were prepared for luciferase assays, according to the manufacturer's instructions (Promega). The results were obtained from at least three sets of transfections and presented as mean values. For the siRNA (small interference RNA) experiments, both PC-3 and LNCaP cells were seeded the night before transfection such that cells reached 30–40 % confluence by the time of transfection. The siRNAs for p300 were chemically synthesized by GenePharm. The siRNA sequence is as follows: p300, 5'-A<sub>2218</sub>AUGCUACUUC-UCGAGGAAACA<sub>2238</sub>-3'. Procyanidin B1 and B2 were purchased from Sigma–Aldrich. Pro-B3 was chemically synthesized by Dr Younghwa Na (Catholic University of Daegu, Gyeongbuk, Korea). Antibodies against acetylated H3 and p300 were purchased from Upstate Biotechnology, and AR and acetylated lysine residues were from Santa Cruz Biotechnology. The AR–GFP (green fluorescent protein) plasmid [GFP–AR (wt)], expressing C-terminally GFP-tagged AR protein, was provided by Dr Toshihiko Yanase (Fukuoka University, Fukuoka, Japan). Both the non-acetylation-mimetic mutant AR–GFP plasmid [GFP–AR (K630/632/633R)] and the acetylation-mimetic mutant AR–GFP plasmid [GFP–AR (K630/632/633Q)] were provided by Dr Masaki Shiota, Dr Akira Yokomizo and Dr Seiji Naito (Kyushu University, Fukuoka, Japan). The plasmids for reporter gene assays [PGL-PSA (ARE)–Luc and FLAG–AR; ARE is androgen-response element, PSA is prostate-specific antigen] have been described previously [21]. The recombinant p300 HAT domain for the *in vitro* acetylation assay was purchased from Upstate Biotechnology. For the GST (glutathione transferase)-fusion protein, AR [aa (amino acids) 507–645] was subcloned into the pGEX4T-1 GST-expression vector (Amersham Pharmacia Biotech). The construct for GST–AR (aa 507–645) was expressed in *Escherichia coli* BL21 cells and affinity-purified using glutathione–Sephadex 4B beads (Amersham Pharmacia Biotech) following the manufacturer's protocol. GST–histone H4 has been described previously [12].

### Clinical specimens

All patient-derived specimens were collected and archived under protocols approved by the IRBs (Institutional Review Boards) of Yonsei University College of Medicine (Seoul, South Korea). Archival tissues in paraffin blocks were retrieved from the Department of Pathology at Yonsei Severance Hospital,

Yonsei University College of Medicine. Specimens were stained with antibodies specific for p300 (Santa Cruz Biotechnology).

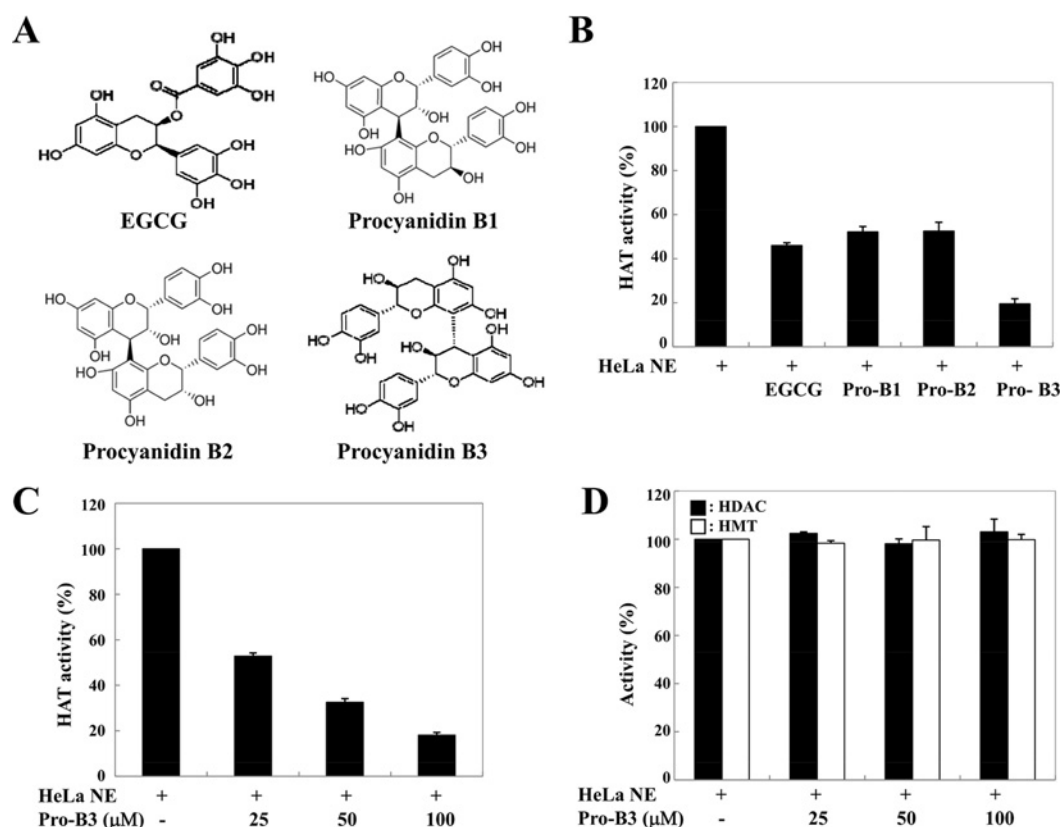
### HAT, HDAC (histone deacetylase) and HMT (histone methyltransferase) assays

HeLa cell NE (nuclear extract) was prepared as described previously [22]. HAT activity and HDAC activity assays were performed using NEs following the manufacturer's protocol (Biovision Biotechnology). For HAT activity assays, IPs (immunoprecipitations) were performed in HeLa NEs using anti-p300, anti-Tip60, anti-CBP and anti-PCAF antibodies (Santa Cruz Biotechnology). Pre-cleared NE was incubated with antibodies overnight with Protein A/G PLUS agarose beads at 4 °C. IPs were collected and washed with HAT assay buffer [50 mM Tris/HCl (pH 8.0), 10 % glycerol and 0.1 mM EDTA]. For recombinant p300 HAT activity assays, HAT activity assays were performed using active p300 (Upstate Biotechnology) following the manufacturer's protocol (Biovision Biotechnology). All samples were counted with a multi-purpose scintillation counter (LS 6500; Beckman). For Figure 2(A), active p300 was incubated with HAT assay buffer [50 mM Hepes (pH 8.0), 10 % glycerol, 1 mM DTT (dithiothreitol), 1 mM PMSF, 10 mM sodium butyrate and 1 µl of [<sup>3</sup>H]acetyl-CoA}, 1 µg of GST–H4 recombinant protein and increasing amounts of Pro-B3 at 30 °C for 1 h. Samples were separated by SDS/PAGE (15 % gels) and analysed by autoradiography. For HMT activity assays, assay buffer and core histones were from the HMT Assay Reagent kit (Upstate Biotechnology). HMT activity assays were performed following the manufacturer's protocol. Core histones were incubated in MTase (methyltransferase) buffer [final concentrations 50 mM Tris/HCl (pH 8.0), 1 mM PMSF and 0.5 mM DTT], 1 µl (0.55 µCi) of [<sup>3</sup>H]AdoMet (S-adenosyl-L-[methyl-<sup>3</sup>H] methionine; Amersham Pharmacia Biotech), and 1 µg of HeLa NE for 1 h at 30 °C in a total volume of 20 µl. The reaction was spotted on to P-81 paper (Upstate Biotechnology) for scintillation counting. P-81 paper was washed three times with 10 % trichloroacetic acid for 15 min. P-81 papers were then washed with 95 % ethanol for 5 min at room temperature (20 °C) and allowed to dry. Dry P-81 papers were counted with a multi-purpose LS 6500 scintillation counter (Beckman).

### RT (real-time) PCR analysis and ChIP (chromatin IP) assays

RNA extraction, RT-PCR and ChIP were performed as described previously [21]. The RT-PCR analysis and quantification were performed with SYBR Green PCR Master Mix reagents on an ABI Prism 7300 Sequence Detection System. The singularity and specificity of amplification were verified by dissociation analysis software. All samples were normalized to human GAPDH (glyceraldehyde-3-phosphate dehydrogenase). Primer sequences for amplification of NKX3-1 RNA were forward, 5'-CTGTCAGCCCCTGAACGG-3' and reverse, 5'-AACCATATCTTCACTTGGGTCTCC-3'. Primer sequences for amplification of the PSA RNA were forward, 5'-AGTCTGAGGAGGTGTTCTGGTG-3' and reverse, 5'-GAGGTCGTGGCTGGAGTCATCA-3'. All reactions were performed in triplicate. Relative expression levels and S.D.s were calculated using the comparative method.

For ChIP assays, LNCaP cells were first transfected with the GFP–AR plasmids indicated and chromatin was isolated as described previously [21]. The antibody against GFP was purchased from Santa Cruz Biotechnology, and histone H3 and acetylated histone H3 antibodies were purchased from



**Figure 1** Pro-B3 has specific anti-HAT activity

(A) Chemical structures of catechin derivatives. (B) Pro-B3 has potent anti-HAT activity toward catechin derivatives. HAT activity was assayed with a colorimetric assay kit. HeLa cell NE provided the source of HAT enzymes. Values are means  $\pm$  S.D. for three independent experiments. (C) Pro-B3 inhibits HAT activity in a dose-dependent manner. HAT activity colorimetric assays were performed with indicated concentrations of B3. (D) Pro-B3 treatment had no effect on either HDAC or HMT activity. HDAC activity was assayed with an HDAC activity colorimetric assay kit. TSA (trichostatin A) was used as a control inhibitor for HDAC activity. Values are means  $\pm$  S.D. for three independent experiments. HMT assays were performed in 30  $\mu$ l reaction mixtures in the presence or absence of Pro-B3, using HeLa cell NEs as the source of enzyme, and then processed for filter-binding assays.

Upstate Biotechnology. Primers used for ChIP analysis for PSA were 5'-CATGTTACATTAGTACACCTTGCC-3' and 5'-TCTCAGATCCAGGCTTGCTTACTGRC-3'.

#### IP and Western blot analysis

Cell extracts were prepared using lysis buffer [1% Nonidet P40, 1% sodium deoxycholate, 0.1% SDS, 0.15 M and 50 mM Tris/HCl (pH 7.4), incubated with the antibodies indicated and then incubated with 20  $\mu$ l of Protein A/G agarose overnight at 4 °C. Co-immunoprecipitated proteins were separated by SDS/PAGE (10% gels), transferred on to nitrocellulose membranes, subjected to immunoblot analysis and visualized by autoradiography.

#### Colony formation and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] assay

The degree of cell proliferation was evaluated by MTT assays. In brief, 10  $\mu$ l of MTT solution (5 mg/ml) was added to each well 4 h prior to the end of the incubation. Formazan crystals formed in the cells were dissolved by adding 100  $\mu$ l of MTT solvent (0.01 M HCl in 10% SDS). The absorbance at 550 nm was measured using a spectrophotometer 24 h after the addition of the MTT solvent. Colony-formation assays were performed using the CytoSelect™ 96-well Cell Transformation Assay kit (Cell Biolabs), according to the manufacturer's instructions.

#### Annexin V staining and FACS analysis

Cells were stained with both Annexin V-FITC and propidium iodide, using the ApoScan Annexin-V FITC Apoptosis Detection Kit (BioBud), according to the manufacturer's protocol. Alternatively, we fixed cells in 70% (v/v) ethanol and stained them with a solution containing RNase A (50  $\mu$ g/ml) and propidium iodide (50  $\mu$ g/ml).

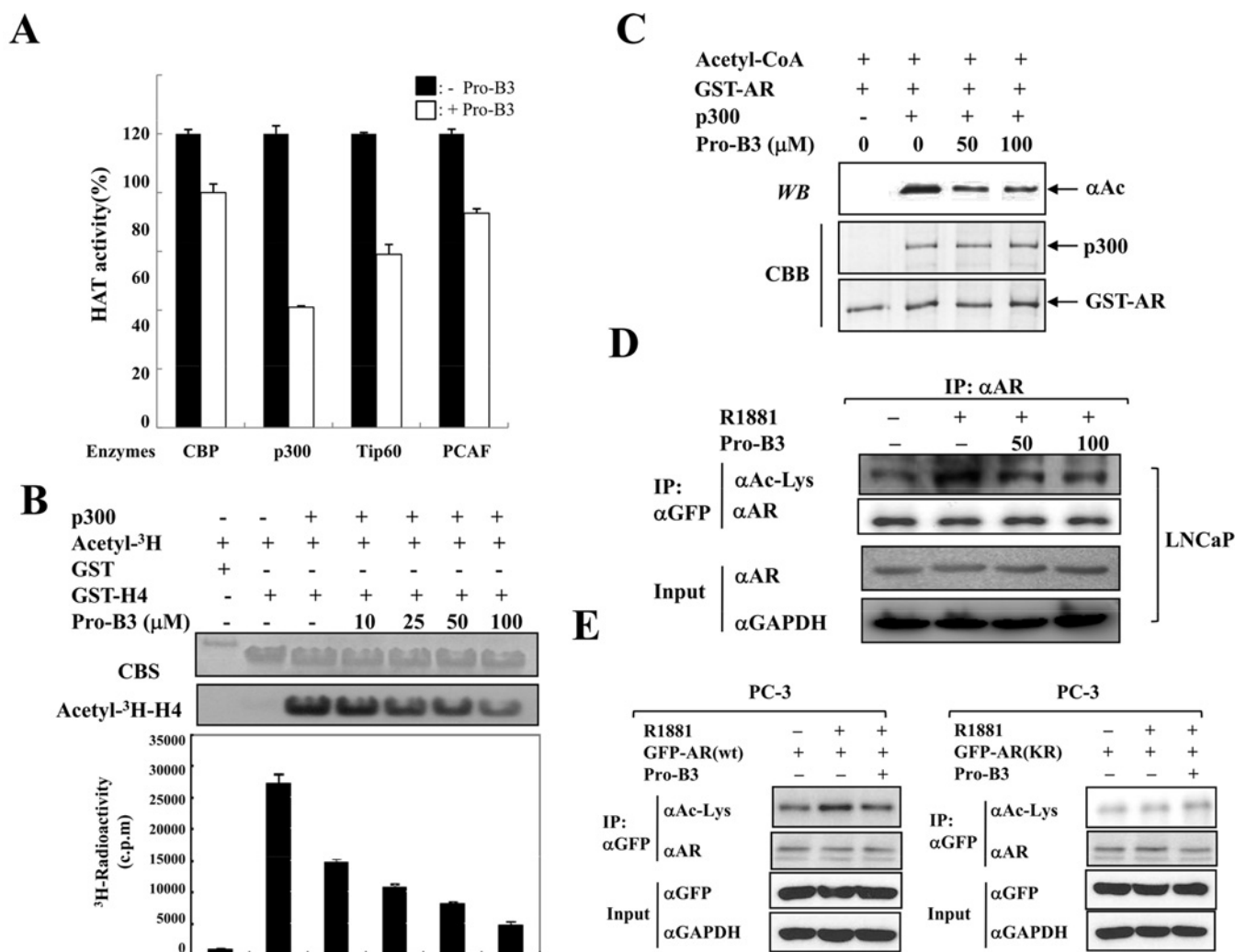
#### Statistical analysis

Statistical analysis was performed using a Student's *t* test with the SPSS program. *P* < 0.05 was considered to be significant.

## RESULTS

#### Pro-B3, a novel HATi, exhibits the highest anti-HAT activity of all catechin derivatives

During an ongoing screen of natural compounds for anti-HAT activity, we found that a methanol extract of grape seeds has potent anti-HAT activity against the HAT p300/CBP [16]. The most abundant polyphenols in grape seeds are procyanidins, which are also found in cereals, fruits and tea. As shown in Figure 1(A), the procyanidin is composed of catechin dimers. Since catechin derivatives possess anti-HAT activity [12,23], we next compared the anti-HAT activity of Pro-B3 to the activities of other catechin derivatives. As shown Figure 1(B),



**Figure 2** Pro-B3 inhibits p300-dependent AR acetylation *in vitro* and *in vivo*

(A) Pro-B3 inhibits p300 HAT activity in a dose-dependent manner *in vitro*. *In vitro* HAT activity assays were performed with GST-H4 tail and p300 HAT enzymes in the presence or absence of Pro-B3, and subsequently processed for fluorography. (B) HAT activity colorimetric assays were performed with immunoprecipitated HAT proteins, as indicated. The results are shown as the percentage of non-treated Pro-B3. (C) Pro-B3 inhibits the p300-mediated AR acetylation *in vitro*. *In vitro* acetylation assays were performed with GST-AR (aa 507–645) in the presence or absence of B3, and subsequently processed for Western blot analysis with an antibody against acetylated lysine. (D) Pro-B3 treatment prevented the ligand-dependent AR acetylation *in vivo*. To assess the levels of endogenous AR acetylation, LNCaP cells were treated with R1881 and/or Pro-B3. The immunoprecipitated proteins were processed for Western blot analysis with the antibodies indicated (top panel). (E) To assess the levels of exogenous AR acetylation, PC-3 cells were transfected with either wild-type GFP-AR or acetylation mutant GFA-AR (K630R/632R/633R) with R1881 and/or Pro-B3. The levels of AR acetylation were assayed by Western blot analysis with the antibodies indicated. Ac-Lys, acetylated lysine; IP, immunoprecipitation; WB, Western blot.

EGCG exhibited HAT inhibitory activities by up to 60 % at 50 μM, consistent with a previous study [12]. Pro-B3 inhibited 90 % of HAT activity in a dose-dependent manner (Figure 1C), and exhibited the highest anti-HAT activity of the procyanidin derivatives, although procyanidin B1 and B2 had levels of anti-HAT activity similar to EGCG (Figure 1B). To examine enzyme specificity, we first tested the effect of Pro-B3 on HDAC and HMT activities. When HeLa NEs were used as a source of HDAC enzymes, deacetylation activity was not affected by the presence of Pro-B3 (Figure 1D). This suggests that Pro-B3 is not a specific inhibitor of HDACs.

#### Pro-B3 inhibits p300-dependent AR acetylation

To examine whether Pro-B3 is a general HATi or a specific HATi, HAT activity was measured for immunoprecipitated p300, Tip60,

PCAF and CBP enzymes. As shown in Figure 2(A), Pro-B3 was found to be an efficient inhibitor of p300 acetyltransferase activity, exhibiting 60 % inhibition compared with the control. Under similar conditions, Pro-B3 only inhibited Tip60, PCAF and CBP acetyltransferase activities by 40 %, 23 % and 20 % respectively. These results suggest that Pro-B3 preferentially inhibits p300 HAT activity. To further validate the B3-mediated selective inhibition of HAT activity of p300, we next analysed radiolabelled GST-H4 protein by SDS/PAGE and fluorography. As shown in Figure 2(B), Pro-B3 strongly quenched p300 HAT activity in a dose-dependent manner, consistent with the results of the HAT activity colorimetric assays. We next examined how Pro-B3 inhibited p300 HAT activities. For this experiment, we studied the kinetics of p300 HAT activity in the presence of Pro-B3 in reactions with changing histone concentrations. We found that  $K_m$  and  $V_{max}$  were decreased with Pro-B3, demonstrating that Pro-B3 does not bind to the active sites of p300, but to

some other site on the enzyme (see Supplementary Figure S1 at <http://www.BiochemJ.org/bj/433/bj4330235add.htm>). These results establish that Pro-B3 uncompetitively inhibits the p300 HAT activity, but not other epigenetic enzymes.

Since AR is acetylated by p300, we next examined whether Pro-B3 directly inhibits p300-mediated AR acetylation. *In vitro* acetylation assays were performed in the presence or absence of Pro-B3, using recombinant GST-AR (aa 507–645) as a substrate. As shown in Figure 2(C), the acetylation of GST-AR was detected in the presence of active p300 protein, but not without p300 protein. Upon Pro-B3 treatment, the p300-induced acetylation was reduced, indicating that Pro-B3 suppresses the acetylation of AR *in vitro*. To confirm the inhibition of p300-mediated AR acetylation by Pro-B3, we assessed the effect of Pro-B3 treatment on androgen R1881-induced acetylation of AR in LNCaP prostate cancer cells. To do this, IP was performed on LNCaP cell lysates with a specific AR antibody and Western blot analysis was used to determine the amount of acetylation at specific lysine residues. As shown in Figure 2(D), AR acetylation was increased in LNCaP cells in the presence of R1881, and the levels of AR acetylation was reversed by Pro-B3 treatment, indicating that Pro-B3 suppresses the acetylation of AR *in vivo*. The lysine residues (Lys<sup>630</sup>/Lys<sup>632</sup>/Lys<sup>633</sup>) of AR within the hinge region are known to be acetylated by p300. We next examined whether androgen-induced AR acetylation is inhibited by Pro-B3 via these lysine residues of AR. For this experiment, PC-3 cells were transfected with GFP-AR (wt) or the non-acetylation mimetic mutant GFP-AR (K630/632/633R) expression plasmids, and the levels of AR acetylation were assessed by Western blot analysis. Androgen efficiently induced the acetylation of GFP-AR (wt), whereas Pro-B3 treatment inhibited the R1881-induced acetylation of AR. On the other hand, neither R1881 nor Pro-B3 treatment had an effect on the acetylation level of GFP-AR (K630/632/633R), indicating that the inhibition of R1881-induced AR acetylation by Pro-B3 treatment is mainly through the lysine residues in the hinge region of AR (Figure 2E). Collectively, these data suggest that Pro-B3 inhibits p300-mediated AR acetylation both *in vitro* and *in vivo*.

### Pro-B3 suppresses R1881-mediated AR transcription in a p300-dependent manner

To measure the effects of Pro-B3 on AR-mediated transcription in LNCaP cells, we used an androgen-dependent reporter vector (pGL3-PSA) containing the ARE and the luciferase reporter gene. LNCaP cells were treated with the synthetic androgen R1881 in the presence or absence of Pro-B3 for 48 h in RPMI 1640 medium containing charcoal-stripped FBS. As shown in Figure 3(A) (left-hand panel), Pro-B3 inhibited agonist-bound AR-mediated transcription, in a dose-dependent manner.

We next performed RT-PCR analysis to determine whether Pro-B3 inhibits R1881-induced transcription of AR-regulated genes. LNCaP cells were exposed to 0, 50 or 100  $\mu$ M Pro-B3 for 18 h, and total RNA samples were isolated. Consistent with the results of the reporter assay, both an androgen-dependent increase and Pro-B3-induced reduction were observed in the mRNA levels of PSA and NKX 3.1 (Figure 3A, right-hand panel). Taken together, these studies indicate that Pro-B3 inhibits the agonist-induced AR-dependent transcription.

Since several HATi from natural substances have already been identified, we compared the relative efficacy of Pro-B3 with the other known HATi. As shown in Figure 3(B), Pro-B3 was the most potent inhibitor of agonist-induced AR transcription among the reported HATi, although the anti-HAT activity of curcumin

was similar to that of Pro-B1. Therefore the tendency of AR-mediated inhibition by HATi seems to be closely related to their HAT activities.

p300 plays a role in androgen-dependent AR transcription. Thus we next asked whether Pro-B3 suppresses androgen-dependent AR transcription via inhibition of p300 activity. As shown in Figure 3(C), siRNA specific for p300 was able to efficiently reduce the level of p300 when used at a concentration of 1 nM. Similar to Pro-B3 treatment, the depletion of p300 dramatically relieved androgen-dependent AR transcription. Interestingly, combined treatment of Pro-B3 with siRNA against p300 further reduced the transcription of AR-target genes, indicating that Pro-B3 suppresses AR transcription via inhibition of p300 activity (Figure 3D).

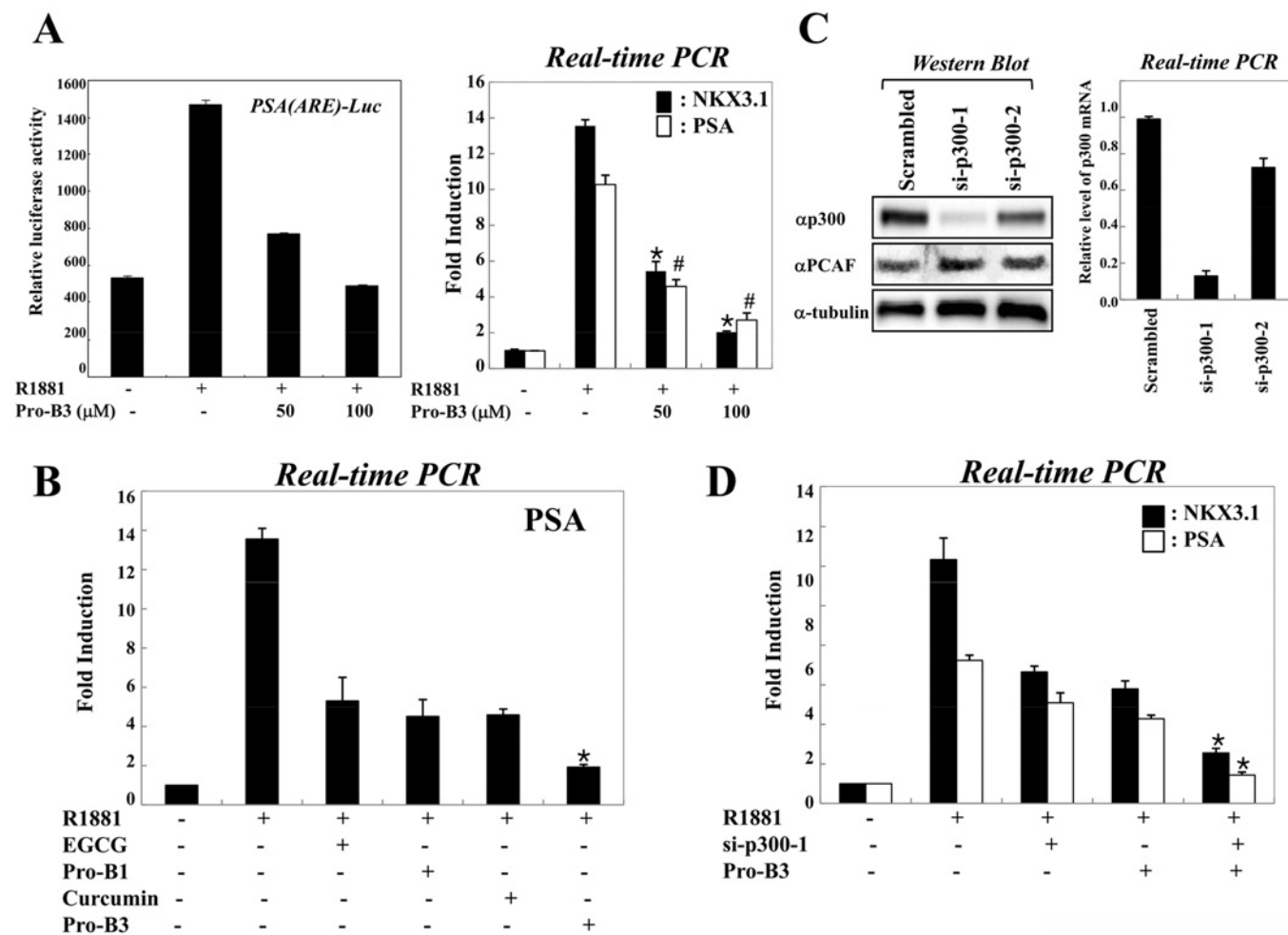
### Pro-B3 inhibits p300-enhanced hormone responsiveness and enhances the antagonist action

Since p300 plays a role in the hormone responsiveness of AR, we next examined whether Pro-B3 inhibits the hormone responsiveness of AR in an acetylation-dependent manner. For this experiment, either GFP-AR (wt) or GFP-AR (K630/632/633R) with pGL3-PSA reporter plasmid and pSG5-p300 plasmid was transiently transfected into PC-3 cells. The p300 further enhanced the GFP-AR (wt)-mediated transcription in the presence of ligand, which was abrogated by Pro-B3 treatment. Interestingly, neither the overexpression of p300 nor Pro-B3 treatment had a significant effect on GFP-AR (K630/632/633R)-dependent transcription, suggesting that p300-mediated AR acetylation is critical for the hormone responsiveness of AR (Figure 4A and Supplementary Figure S2A at <http://www.BiochemJ.org/bj/433/bj4330235add.htm>).

Given the knowledge that the HATi Pro-B3 inhibits the p300-enhanced hormone responsiveness of AR, we next tested the possibility that Pro-B3 enhances antagonist action. As shown in Figure 4(B), overexpression of p300 in LNCaP cells resulted in a substantial increase in the transcription of PSA with R1881 treatment, compared with LNCaP cells with R1881 alone. As expected, flutamide treatment efficiently suppressed the R1881-induced transcription of the PSA gene. Importantly, Pro-B3 treatment (50  $\mu$ M) repressed transcription of both the NKX3.1 and PSA genes to a level similar to flutamide treatment. More importantly, co-treatment of Pro-B3 with flutamide increased the inhibition of p300-mediated AR transcription, suggesting that Pro-B3 enhances the antagonistic activity of flutamide via suppression of p300 activity (Figure 4B).

The enhancement of antagonistic action by Pro-B3 led us to ask whether the relative level of p300 is critical for the inhibition of AR-mediated transcription by Pro-B3 and hormone responsiveness of AR. As shown in Figure 4(C) and Supplementary Figure S2(B), knock down of p300 further potentiated the suppression of GFP-AR (wt)-mediated transcription, but not in the case of mutant GFP-AR (K630/632/633R). Furthermore, combined treatments of si-p300 with either flutamide or Pro-B3 efficiently inhibited GFP-AR (wt)-mediated transcription, compared with si-p300 alone. As expected, GFP-AR (K630/632/633R)-mediated transcription was not affected upon silencing of p300. These data suggest that the overall level of p300 is a key determinant of antagonist activity.

We next asked whether p300 mediates the acetylation of AR, as well as the histone hyperacetylation, which is required for AR-mediated transcriptional activation, and subsequently leads to the up-regulation of PSA transcription. For this experiment, either GFP-AR (wt) or GFP-AR (K630/632/633R) was transfected



**Figure 3** Pro-B3 suppresses R1881-mediated AR transcription in a p300-dependent manner

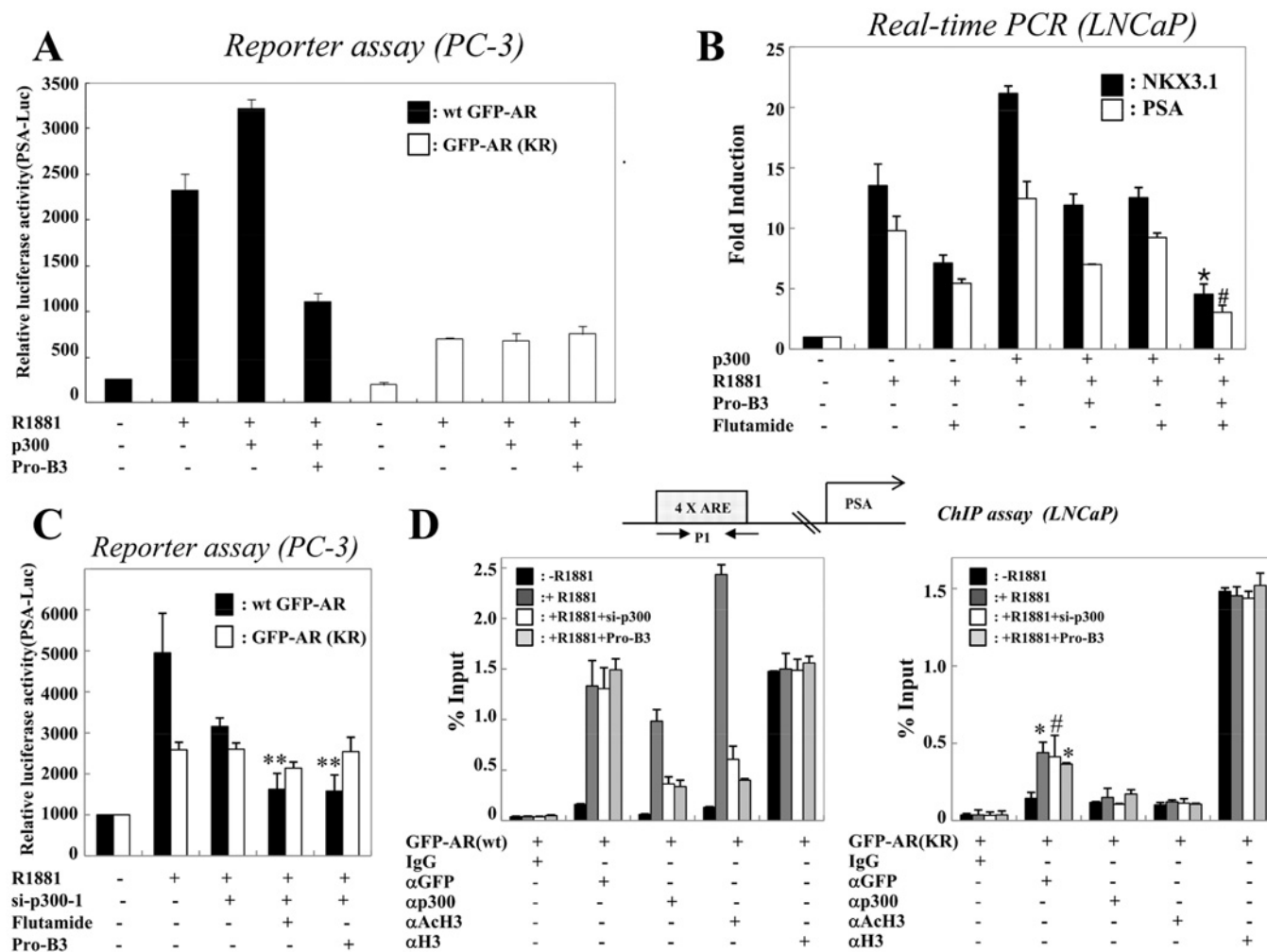
(A) Pro-B3 inhibited R1881-induced AR-dependent promoter activity in LNCaP cells. LNCaP cells were transfected with an AR-binding site-driven luciferase reporter plasmid [PGL-PSA (ARE)–Luc] and treated with R1881 and/or increasing amounts of Pro-B3. Whole-cell extracts were used in luciferase assays. Results are presented as the means of two independent experiments performed in triplicate (left-hand panel). Total RNA was prepared from each sample and used for quantitative RT-PCR to measure the expression of the AR target genes, PSA and NKX3.1. As controls, the level of GAPDH mRNA was also measured by quantitative RT-PCR in the same batch of RNA samples. The levels of transcription were shown as the fold-induction compared with the level of transcription in the absence of R1881, which was set as 1 (right-hand panel). \* $P < 0.01$  compared with R1881; # $P < 0.05$  compared with R1881. (B) Pro-B3 is the strongest inhibitor of R1881-dependent AR transcription of the known HAT inhibitors. LNCaP cells were treated with 50  $\mu$ M HAT inhibitors and the effect on transcriptional activation of PSA was analysed by quantitative RT-PCR analysis. Values are the means  $\pm$  S.D. for at least three independent experiments. \* $P < 0.01$  for Pro-B3 compared with curcumin. (C) Knockdown of p300 with specific siRNA. LNCaP cells were transfected with control (scrambled) siRNA or siRNAs against p300 at the concentrations indicated. At 3 days after treatment, whole-cell extracts were prepared and the level of p300 was determined by Western blot analysis with the p300 antibody. Western blot results for PCAF and tubulin served as specificity controls. (D) Pro-B3 suppresses the AR transcription via inhibition of p300 activity. LNCaP cells were first treated with siRNA against p300. The cells were then treated with 1 nM R1881 and/or 50  $\mu$ M B3 for 12 h and the level of transcription of NKX3.1 and PSA was analysed by quantitative RT-PCR analysis. Values are means  $\pm$  S.D. for at least three independent experiments. \* $P < 0.01$  for Pro-B3+si-p300 compared with si-p300.

into LNCaP cells in the presence or absence of si-p300 or Pro-B3, and then ChIP assays were performed using anti-GFP, p300, acetylated histone H3 or histone H3 antibodies. To more precisely measure the recruitment of AR and p300, we used RT-PCR analysis. The results in Figure 4(D) showed that R1881 treatment led to a significant increase in the binding of GFP–AR (wt) to the enhancer region of PSA. Although Pro-B3 or si-p300 treatment had little effect on the recruitment of GFP–AR (wt), the recruitment of p300 was reduced by treatment with si-p300 or Pro-B3. Consequently, the level of histone H3 acetylation was greatly increased by R1881 and, conversely, Pro-B3 treatment reversed the R1881-induced histone H3 hyperacetylation. Importantly, neither Pro-B3 nor si-p300 treatment had an effect on the recruitment of either p300 or histone acetylation in the presence of GFP–AR (K630/632/633R), suggesting that p300-mediated

AR acetylation is required for the AR-mediated transcriptional activation.

#### Pro-B3 specifically inhibits acetylation-dependent prostate cancer cell growth

Since AR acetylation is closely correlated with prostate cancer cell growth, we next examined the effect of Pro-B3 on this function of AR. To do this, we performed MTT assays in LNCaP, HEK (human embryonic kidney)-293 and NIH 3T3 cell lines. As shown in Figure 5(A), Pro-B3 treatment increased LNCaP cell death by approx. 95 % at 100  $\mu$ M, but did not affect either NIH 3T3 or HEK-293 cells. This indicated that B3 treatment reduces cell viability rather than causing cytotoxicity.



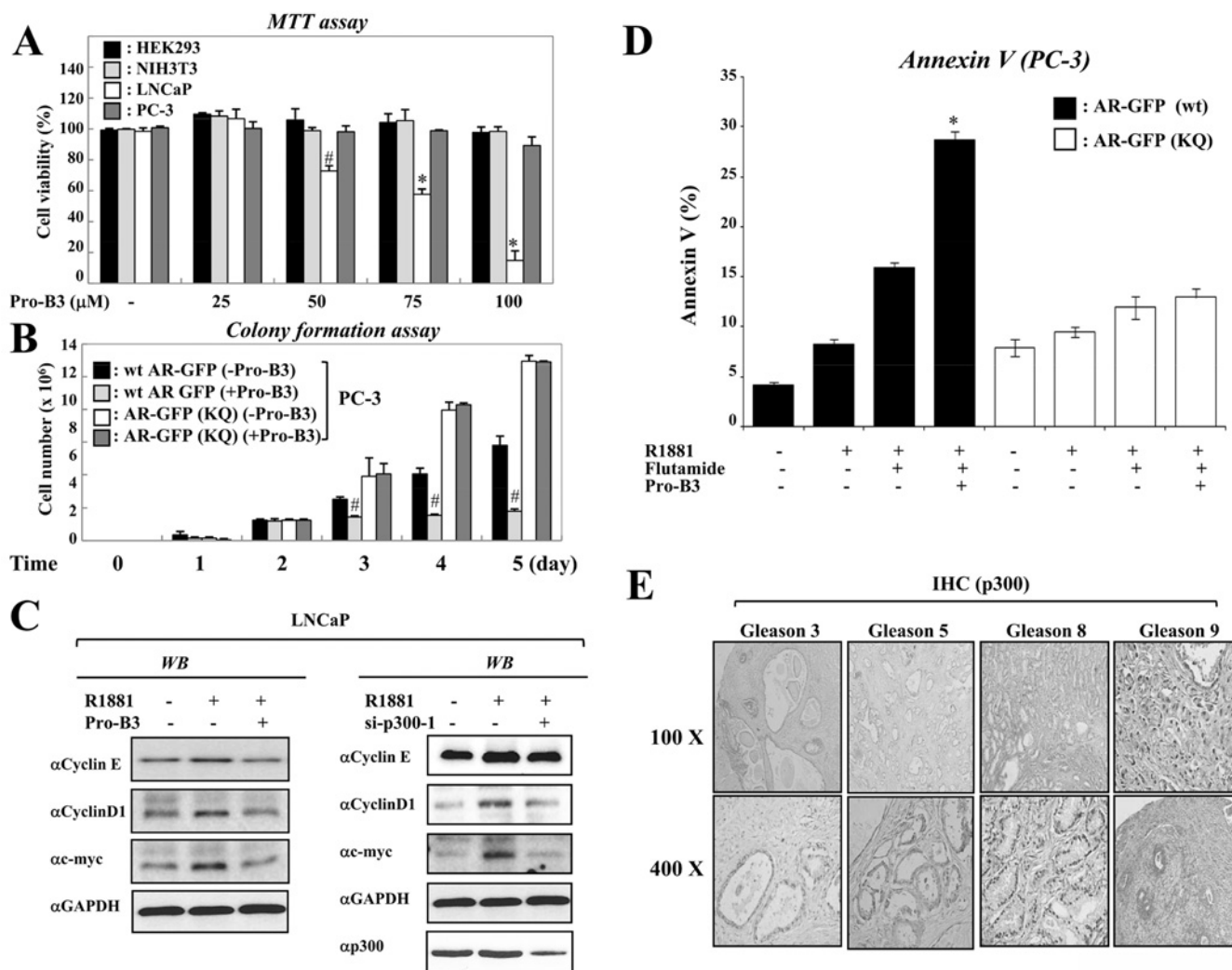
**Figure 4** Pro-B3 inhibits p300-enhanced hormone responsiveness and enhances antagonist action

(A) Pro-B3 suppressed the hormone responsiveness of AR via inhibition of p300-mediated AR acetylation. PC-3 cells were transfected with the indicated AR–GFP expression plasmids and PGL-PSA (ARE)—Luc reporter plasmid. The cells were then treated with 1 nM R1881 and/or Pro-B3 (50  $\mu$ M) for 12 h. Whole-cell extracts were used in luciferase assays. Results are presented as the means of two independent experiments performed in triplicate. KR, K630/632/633R. (B) Pro-B3 enhanced the antagonist activity of flutamide via suppression of p300 activity. LNCaP cells were first transfected with either p300 expression plasmid or control psg5.1 control vector. The cells were then treated with R1881 (1 nM), flutamide (10  $\mu$ M) or B3 (50  $\mu$ M), individually or in combination, for 12 h and the levels of transcription from PSA and NKX3.1 were analysed by quantitative RT-PCR analysis. Values are means  $\pm$  S.D. for at least three independent experiments. \* $P$  < 0.01 for flutamide+Pro-B3 compared with flutamide; # $P$  < 0.01 for flutamide + Pro-B3 compared with flutamide. (C) The relative level of p300 is critical for the inhibition of AR-mediated transcription by B3 and hormone responsiveness of AR. PC-3 cells were transfected with the indicated AR–GFP expression plasmids and PGL-PSA (ARE)—Luc reporter plasmid. The cells were then treated with R1881 (1 nM), flutamide (10  $\mu$ M) or Pro-B3 (50  $\mu$ M), individually or in combination, for 12 h. Whole-cell extracts were used in luciferase assays. Results are presented as the means of two independent experiments performed in triplicate. \*\* $P$  < 0.05 compared with si-p300. (D) Pro-B3 inhibits the p300-dependent acetylation of both AR and histone tails, and subsequent chromatin targeting of p300. LNCaP cells were transfected with indicated AR–GFP expression plasmids with or without si-p300. The cells were then treated with 1 nM R1881 and/or Pro-B3 (50  $\mu$ M) for 12 h, and the recruitment of GFP–AR proteins and p300 to the enhancer region of the PSA gene was then determined by ChIP assays using antibodies, as indicated. The results were analysed by RT-PCR and are shown as the percentage of input. \* $P$  < 0.01 for +R1881 or +R1881+Pro-B3 compared with –R1881; # $P$  < 0.05 for +R1881+si-p300 compared with –R1881.

To further examine the role of AR acetylation on prostate cancer cell proliferation, PC-3 cells were transfected with either GFP–AR (wt) or acetylation-mimicking GFP–AR (K630/632/633Q) and/or Pro-B3 in the absence of R1881, and the relative rate of cell proliferation was assessed by colony-formation assays. The overexpression of acetylation-mimicking GFP–AR (K630/632/633Q) enhanced cell proliferation compared with GFP–AR (wt). Pro-B3 treatment failed to suppress the increase of cell proliferation by GFP–AR (K630/632/633Q), whereas it efficiently blocked wild-type AR–GFP-enhanced cell proliferation (Figure 5B). These data indicate that Pro-B3 inhibits prostate cancer cell proliferation by blocking AR acetylation.

Next, we examined the effect of Pro-B3 on cell proliferation by assessing the expression of growth control genes. Western blot analysis of LNCaP cell lysates demonstrated that cyclin D1 and cyclin E protein levels were both decreased by Pro-B3 treatment. In addition, si-p300 treatment blocked the R1881-induced expression of both genes. These results suggest that the enhanced growth advantage correlated with increased activation of cell-cycle control genes is regulated by AR acetylation (Figure 5C and Supplementary Figure S3 at <http://www.BiochemJ.org/bj/433/bj4330235add.htm>).

As AR acetylation mimetic mutants are shown to be resistant to apoptosis [9], we next examined the effect of Pro-B3 on



**Figure 5** Pro-B3 specifically inhibits the acetylation-dependent prostate cancer cell growth

(A) Pro-B3 inhibits the prostate cancer cell viability and proliferation by blocking AR acetylation. Pro-B3 treatment efficiently decreased viability of LNCaP cells. LNCaP, PC-3, HEK-293 and NIH 3T3 cells were treated with serum-free medium containing increasing amounts of Pro-B3, for 72 h. Cell viability was determined by MTT assays, as described in the Materials and methods section. Values are means  $\pm$  S.D. for three independent experiments (top panel). \* $P < 0.01$ ; # $P < 0.001$  compared with without B3. (B) The anti-proliferative effects of Pro-B3 on LNCaP cells. LNCaP cells were transfected with either wild-type (wt) AR-GFP or mutant (mt) AR-GFP and/or 50  $\mu$ M Pro-B3 and seeded in soft agar. Colony numbers and sizes (percentage of colonies with  $>100$  cells) are shown at day 4 (bottom panel). \* $P < 0.01$ ; # $P < 0.001$  for wild-type AR-GFP+Pro-B3 compared with wild-type AR-GFP. (C) Pro-B3 inhibits the expression of cell-cycle control genes. LNCaP cells were treated with either 50  $\mu$ M Pro-B3 or si-p300 in the presence or absence of R1881. The cell lysates were analysed by Western blot using antibodies, as indicated. (D) HATi Pro-B3 efficiently promotes cellular apoptotic responses. PC-3 cells were transfected with GFP-AR (wild-type) or GFP-AR (KQ) in the presence or absence of Pro-B3. After transfection for 48 h, cells were stained with FITC-conjugated Annexin V. The positive cells were measured by flow cytometry. Values are the means  $\pm$  S.D. for three independent experiments. KQ, K630/632/633Q. (E) p300 is highly expressed in high Gleason grade prostate cancers (PCa). Specimens of Gleason grade 3, 6 and 9 PCa were stained for p300. The data are representative of three separate specimens for each classification. IHC, immunohistochemistry; WB, Western blot.

apoptosis in prostate cancer cells using the ApoDETECT Annexin V-FITC Kit, which detects early stages of apoptosis. Apoptotic cells can be recognized and distinguished from necrotic cells using flow cytometry after double staining by Annexin V-FITC and propidium iodide. As shown in Figure 5(D), combinatorial treatment of Pro-B3 with flutamide induced significant apoptosis in GFP-AR (wt)-transfected PC-3 cells, compared with flutamide alone. However, flutamide treatment with Pro-B3 did not show a synergistic effect on apoptosis in GFP-AR (K630/632/633Q)-transfected PC-3 cells (Supplementary Figure S4 at <http://www.BiochemJ.org/bj/433/bj4330235add.htm>). These results suggest that the HATi Pro-B3 inhibits AR acetylation-dependent prostate cancer cell growth and

synergistically promotes cellular apoptotic responses with flutamide in prostate cancer cells.

Since our data consistently showed the critical roles of p300 on AR acetylation-dependent prostate cancer cell growth, we next sought to investigate the pathological relevance of p300 in prostate cancer development. To this end, we examined the level of p300 expression in Korean prostate cancer tissues by immunohistochemistry. As shown in Figure 5(E), the expression of p300 was significantly higher in high-grade tumours with Gleason grade 9 compared with low-grade tumours with Gleason grades 3 and 5, confirming a previous report [14]. These data demonstrated the pathological relevance of p300 in the progression of prostate cancer.



## DISCUSSION

In the present study, we have shown for the first time that procyanidins possess inhibitory activities against HAT, and that Pro-B3 possesses the highest anti-HAT activity among the catechin derivatives. Pro-B3 is a catechin dimer found in barley and beer. During a screen for HATi from natural substances, we found that grape seed extract exhibits high anti-HAT activity. Grape seed extract also exhibits strong antitumour activity and contains high levels of phenolic derivatives, especially dimers, trimers and oligomers of catechin, epicatechin and gallate. Among them, Pro-B3 had the highest anti-HAT activity, especially against p300 HAT activity. Interestingly, the inhibitor activity of Pro-B3 is approx. 2-fold higher than both B1 and B2, suggesting that the position of the catechin monomer is a key factor for anti-HAT activity. The anti-HAT activity of catechin derivatives is likely to be attributed to the structural feature of catechin, since we failed to detect anti-HAT activity from other flavonoid derivatives, including Sophoretin, Quercetin and Meletin (results not shown). Thus the anti-cancer or cancer-preventive effects of catechin derivatives in grape seed may correlate with anti-HAT activity.

In addition to histones, more than 70 non-histone proteins are reversibly acetylated by HAT in relation to apoptosis, inflammation, cell-cycle factors, co-activators and structural proteins [24]. This acetylation modulates multiple cellular events, including protein stability, nuclear translocation, protein–protein interaction and cell growth. The nuclear hormone receptor AR is also acetylated by p300, PCAF and Tip60, and acetylation of AR regulates co-regulator recruitment and growth properties of the receptor in cultured cells and *in vivo*. Previous studies have shown that the overexpression of AR acetylation mimetic mutants leads to reduced apoptosis and an enhanced growth rate with the increased transcription of cell-cycle-regulated genes [9]. Furthermore, point mutations of lysine residues within the acetylation motif of AR have been identified in prostate cancer tissues. This collectively suggests that AR acetylation is a promising target for the development of more effective antagonists of prostate cancer. *In vivo* acetylation assays have demonstrated that the R1881-induced acetylation of AR is efficiently suppressed by Pro-B3 treatment. Interestingly, Pro-B3 treatment inhibited p300-dependent wild-type AR–GFP transcription, but not that of an acetylation mutant of AR–GFP, suggesting that p300-mediated AR acetylation is crucial for AR transcriptional activation. Tip60-mediated AR acetylation is a critical event for AR translocation [7,8]. In addition, Pro-B3 also inhibited the HAT activity of Tip60, although less than that of p300. However, we failed to detect a change of ligand-dependent translocation of AR upon Pro-B3 treatment (Supplementary Figure S5 at <http://www.BiochemJ.org/bj/433/bj4330235add.htm>), indicating that the inhibitory effect of Pro-B3 on the AR function is not related to the Tip60-dependent pathway. p300 acetylates the lysine residue at 630 of AR and regulates coactivator–corepressor complex binding, altering expression of growth control genes to promote aberrant cellular growth *in vivo* [25]. On the other hand, the alteration of the lysine residue at 630 of AR does not affect the distribution of AR, indicating p300-mediated AR acetylation is not correlated with subcellular localization of AR [9]. Thus it is plausible that Pro-B3 preferentially inhibits the p300-dependent transcription of AR, rather than inhibiting transcription mediated by Tip60.

In the present study, we provide evidence that Pro-B3, a HATi, inhibits AR acetylation and suppresses AR-dependent prostate cancer cell growth. Similar to HDAC inhibitors, the inhibition of histone and non-histone protein acetylation can be exploited for the development of new anticancer drugs. It seems contradictory

that both HATi and HDAC inhibitors have the same effect, since these enzymes have opposing catalytic actions. However, the molecular mechanisms of HATs and HDACs are complicated and are still being explained, and the molecular basis of reversible acetylation in tumorigenesis are not likely to be simple. Until now, the deregulation of HATs has been associated with several diseases, such as cardiac hypertrophy, asthma and cancer. In all of these diseases, the cellular histone and non-histone proteins are hyperacetylated. Therefore the selective and signalling-dependent interference of target protein acetylation by HATi, like Pro-B3, will enhance our understanding of acetylation events in cellular function and may lead to a new class of therapeutic or chemopreventive drugs.

Partial agonist activity of an antagonist is a potential explanation for the clinical observation of hormone-withdrawn syndrome [26–28]. Given that the functional dependence of flutamide on HDAC corepressor has been demonstrated [21], relative p300 HAT activity may be a critical point in the efficacy of prostate cancer therapy. In fact, p300 expression in high-grade tumours was shown to be higher compared with low-grade tumours, and has prognostic values in predicting long-term prostate cancer biochemical recurrence-free survival, demonstrating that p300 expression in prostate cancer tissue may be a useful marker for predicting progression [14]. In addition, our data from immunohistochemistry analysis also confirmed the pathological relevance of p300 expression with prostate cancer progression. An important finding in the present study was that Pro-B3 treatment potentiated the antagonist action of flutamide via suppression of HAT. Indeed, several studies have demonstrated that the overexpression of coactivators, including p300, results in decreased antagonist activities of flutamide [14,21,29]. The importance of balance between HDAC corepressors and HAT coactivators has been emphasized in the control of AR protein function [21]. The balance between HDAC and HAT protein seems to determine the levels of histone and AR acetylation and subsequent transcription. Consistent with this notion, our results also demonstrate that the combined treatment of Pro-B3 and flutamide maximizes the efficacy of the antagonist activity. Furthermore, as shown using the acetylation mutant GFP–AR, the inhibitory effect of Pro-B3 on the growth and survival of prostate cancer cells is mainly attributed to the acetylation of AR. In this regard, we have reported the presence of anti-p300 activities in natural compounds including *Rosa rugosa* [30] and allspice extracts [31] which efficiently inhibit AR acetylation. In these reports, both extracts were found to inhibit agonist-dependent AR activation. Intriguingly, similar to Pro-B3, *Rosa rugosa* extracts enhanced antagonist-dependent inhibition of AR transcription, which resulted in reduced prostate cancer cell growth. Thus developing drugs with increased ability to inhibit the AR–p300 interaction or AR acetylation is likely to improve the efficacy of current anti-hormone therapies. In conclusion, the present study raises the possibility that efficient interference of AR acetylation by HATi could lead to a new class of antagonists for the treatment of prostate cancer.

## AUTHOR CONTRIBUTION

KyungChul Choi, SiYong Park, Ah-Reum Sung and Yoo-Hyun Lee designed and performed experiments, and analysed the data with the help of Beom Jin Lim. Masaki Shiota, Akira Yokomizo and Seiji Naito generated various GST–AR plasmids. Younghwa Na and Ho-Geun Yoon directed the project and wrote the manuscript.

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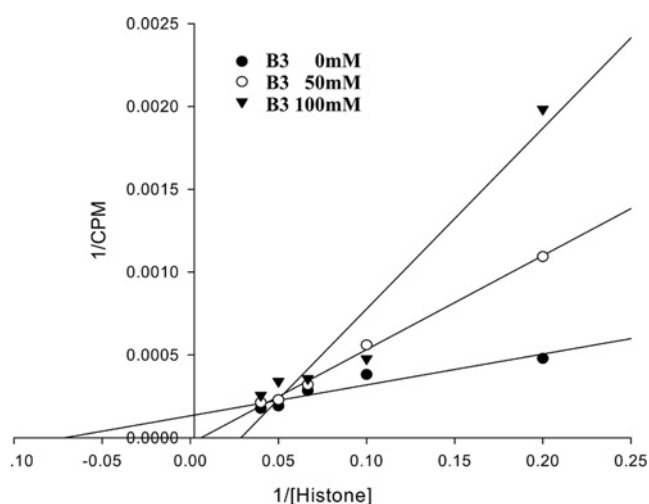
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## SUPPLEMENTARY ONLINE DATA

# Procyanidin B3, an inhibitor of histone acetyltransferase, enhances the action of antagonist for prostate cancer cells via inhibition of p300-dependent acetylation of androgen receptor

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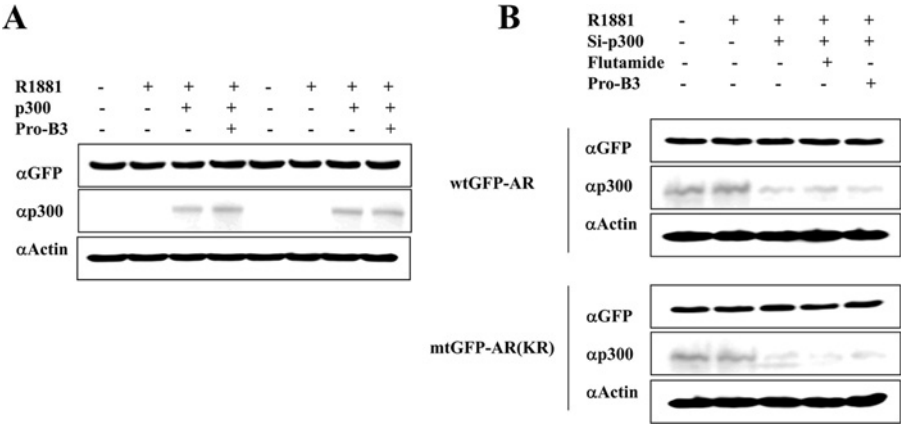


**Figure S1 Pro-B3 uncompetitively inhibits the p300 HAT activity**

Lineweaver–Burk plot showing the effect of Pro-B3 on p300-mediated acetylation of core histones. HAT assays were carried out with a fixed concentration of histones (8 pM) and increasing concentration of [<sup>3</sup>H]acetyl CoA in the presence (50 and 100 μM) or absence of Pro-B3. The results were plotted using Sigma Plot Version 10.0 (SYSTAT Software).

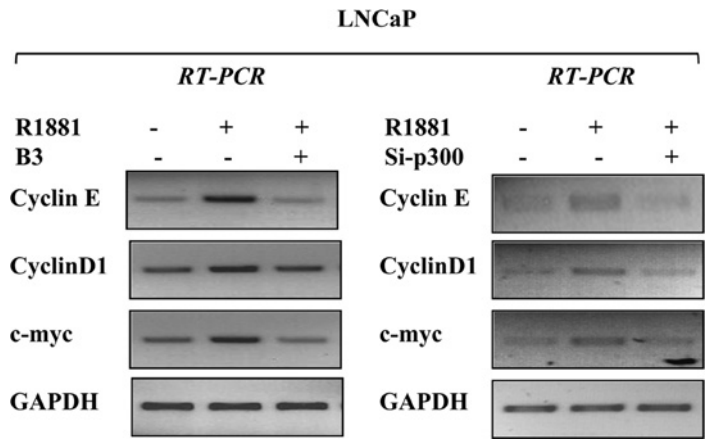
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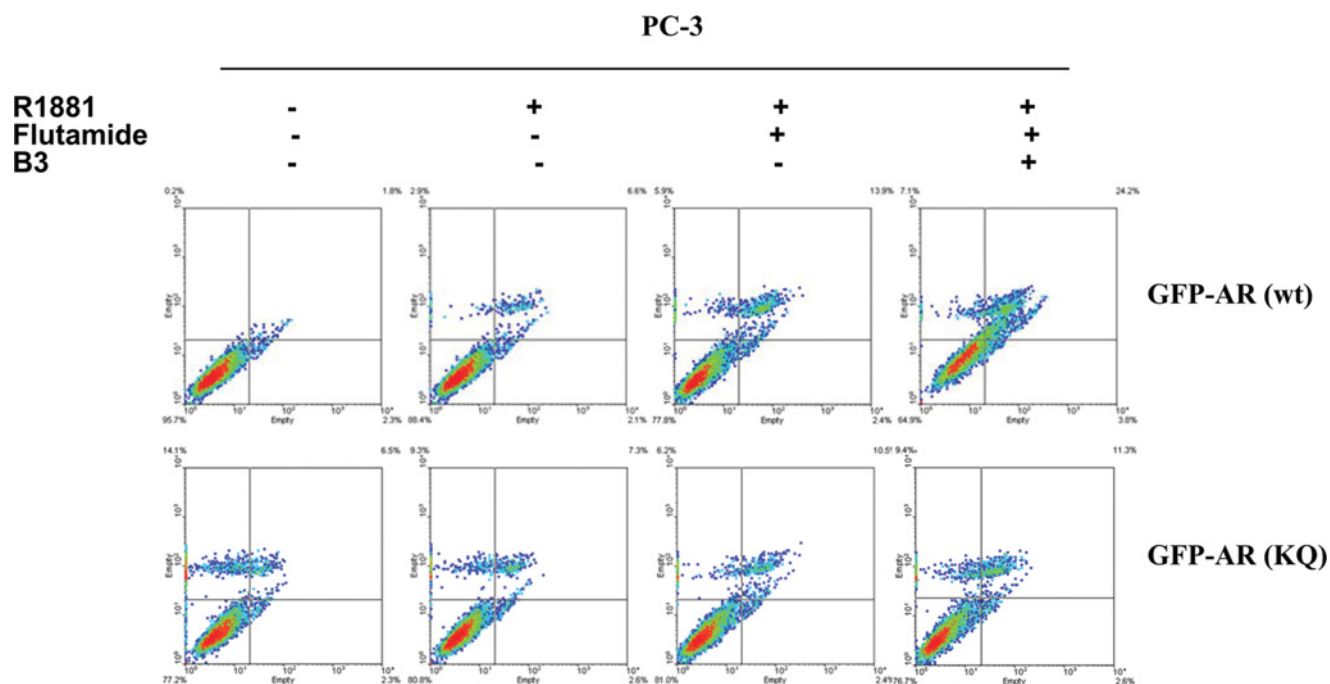
**Figure S2 Western blot analysis for the wild-type and mutant AR–GFP expression plasmids**

PC-3 cells were transfected with indicated GFP–AR (wt) or GFP–AR (mutant) expression plasmids. The cell lysates were analysed by Western blot using antibodies, as indicated.



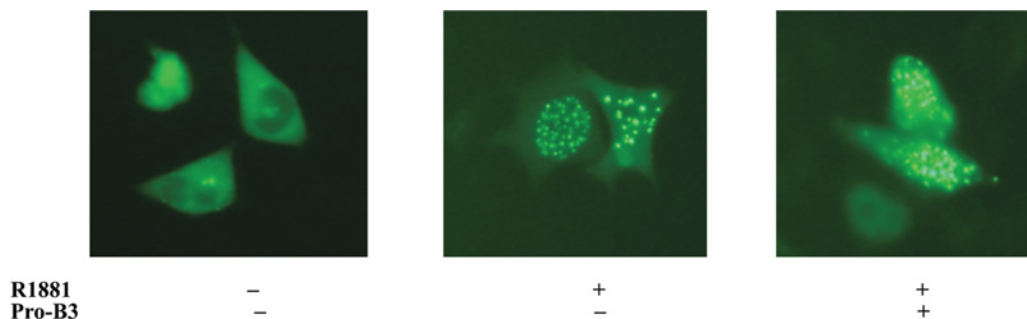
**Figure S3 Pro-B3 inhibits the expression of cell-cycle control genes**

LNCaP cells were treated with either Pro-B3 or si-p300 in the presence or absence of R1881. The cell lysates were analysed by RT-PCR, as indicated.



**Figure S4 Pro-B3 efficiently promotes cellular apoptotic responses with antagonist**

PC-3 cells were stained with FITC-conjugated with Annexin V. The cells were measured by flow cytometry. KQ, K630/632/633Q.



**Figure S5 The effect of Pro-B3 treatment on the cellular localization of AR**

LNCaP cells were transfected with GFP-AR (wt) in the presence or absence of R1881 and/or Pro-B3 as indicated and stained with DAPI (4',6-diamidino-2-phenylindole). The stained cells were observed by fluorescence microscopy.